Fast and cAMP-Sensitive Mode of Ca^{2+}-Dependent Exocytosis in Pancreatic β-Cells

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The fast component (mode 1) of Ca^{2+}-dependent exocytosis in pancreatic β-cells, unlike that in adrenal chromaffin cells, is regulated by cytosolic ATP in a concentration-dependent manner. This action of ATP is apparent within 3 min and does not require ATP hydrolysis; rather, it requires the production of cAMP by adenylate cyclase. Moreover, the effect of cAMP is ATP dependent, as revealed by the observation that the fast component of exocytosis is facilitated by ATP, even in the presence of a saturating concentration of cAMP (200 μmol/l). Thus, the amplitude of mode-1 exocytosis depends quadratically on the cytosolic ATP concentration and is facilitated by ATP, even in the absence of an increase in the concentration of cAMP. Given that high glucose concentrations increase the cytosolic ATP concentration, glucose-induced insulin secretion likely involves this action of ATP on mode-1 exocytosis, together with its effect on ATP-dependent K^+ channels. In contrast to the fast component of exocytosis, the slow component (mode 2) of this process is independent of cAMP and ATP and can account for the slow component of insulin secretion, which does not require these nucleotides. *Diabetes* 51 (Suppl. 1):S19–S24, 2002

Glucose induces insulin secretion from pancreatic β-cells as a result of its metabolism and the associated production of ATP in these cells (1–5). The increase in the cytosolic concentration of ATP triggers the closure of ATP-sensitive K^+ channels (K_{ATP} channels), which, in turn, results in cell depolarization (6,7) and activation of voltage-dependent Ca^{2+} channels. The consequent increase in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$) promotes the exocytosis of insulin secretory granules. This sequence of events is referred to as the K_{ATP} channel–dependent mechanism of glucose-induced insulin secretion.

We have recently shown that cytosolic ATP directly enhances the major, fast component of Ca^{2+}-dependent exocytosis of insulin granules, and that this effect of ATP requires the activation of cAMP–dependent protein kinase (PKA) (8). ATP acts at the "postpriming" step of exocytosis, as indicated by the observation that adenosine-5′-(γ-thio)triphosphate (ATP-γ-S), a nonhydrolyzable analog of ATP, does not block exocytosis, but rather augments it. Thus, acting in concert with the K_{ATP} channel–dependent mechanism during glucose stimulation, the cytosolic concentration of ATP directly regulates the postpriming step of insulin exocytosis. We have now confirmed our previous observations (8) and describe in more detail the actions of ATP on various aspects of insulin exocytosis.

Sensing of ATP by the exocytotic machinery in β-cells. With the use of amperometric detection of secretion from serotonin-loaded pancreatic β-cells, we have shown that ATP in the patch pipette markedly facilitates exocytosis within 3 min after the onset of whole-cell perfusion (Fig. 1*A* and *B*). Given that exocytosis was triggered by photolysis of caged-Ca^{2+} compounds in these studies, the action of ATP cannot be ascribed to an indirect effect on voltage-gated Ca^{2+} channels (9,10). In contrast, identical experiments with adrenal chromaffin cells revealed little effect of ATP within 3 to 5 min after the onset of whole-cell perfusion at room temperature (Fig. 1*C* and *D*), consistent with the results of a previous study based on measurement of capacitance (11). Thus, unlike that of other cell types, the exocytotic machinery of β-cells appears to be sensitive to cytosolic ATP, as has been confirmed in a large number of experiments (Fig. 2*A*). In these experiments, simultaneous measurement of capacitance also revealed a significant facilitation of the capacitance increase by ATP, as previously described by others (12), although this effect of ATP was more variable and of a smaller extent than that detected by amperometric measurements. This difference is likely attributable to the fact that capacitance measurements reflect both exocytosis and endocytosis, rendering the quantitative analysis of exocytosis with this technique difficult in β-cells.

Secretion was triggered by rapid and large increases in Ca^{2+} generated by the photolysis of caged-Ca^{2+} compounds in our previous studies in order to examine the exocytotic steps downstream of Ca^{2+} binding to putative Ca^{2+} sensor molecules. Without such an approach, one cannot exclude a possible effect of the test compound (ATP) on Ca^{2+}-signaling mechanisms. The kinetics of exocytosis are predicted to be 10 to 100 times faster under these experimental conditions than under physiological conditions, both as a result of the larger increases in $[Ca^{2+}]_i$ compared with those that are induced in vivo (13) and because Ca^{2+} appears to act cooperatively on the Ca^{2+} sensors (8). Its predicted time course of ~100 s suggests that ATP-dependent exocytosis contributes predominantly to the first phase of glucose-induced insulin secretion.

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Accepted in revised form 8 June 2001.

AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; ATP-γ-S, adenosine-5′-(γ-thio) triphosphate; $[Ca^{2+}]_i$, cytosolic concentration of Ca^{2+}; cAMP-GEF, cAMP-regulated guanine nucleotide exchange factor; K_{ATP} channel, ATP-sensitive K^+ channel; PKA, cAMP–dependent protein kinase; PKI, protein kinase inhibitor; Rp-cAMP, adenosine 3′,5′-monophosphothioate.

The symposium and the publication of this article have been made possible by an unrestricted educational grant from Servier, Paris.

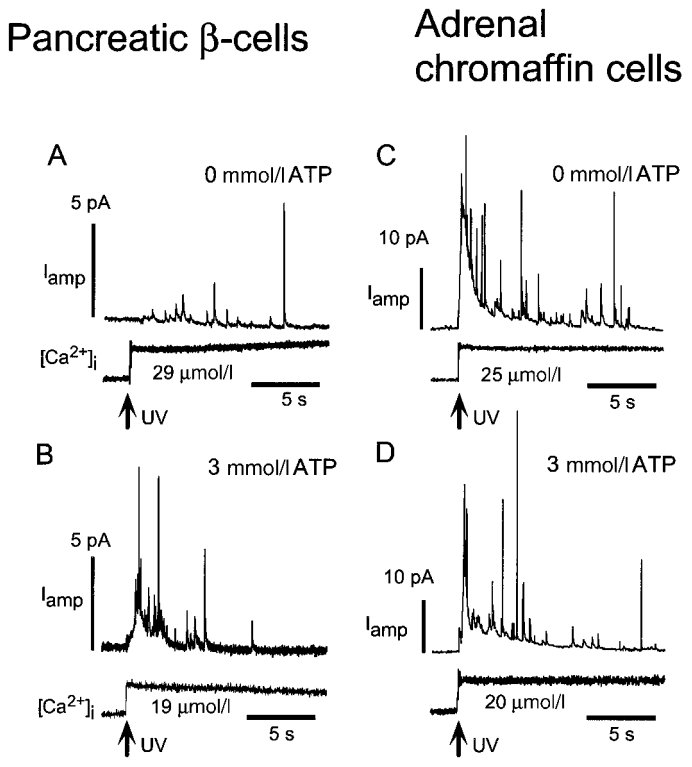

FIG. 1. Comparison of the effects of cytosolic ATP on exocytosis in pancreatic β-cells (*A* and *B*) and adrenal chromaffin cells (*C* and *D*). Exocytosis was triggered by a rapid increase in $[Ca^{2+}]_i$ (lower traces) induced by photolysis of caged-Ca^{2+} compounds [nitrophenyl-EGTA in (*A*) and (*B*), and dimethoxynitrophenyl-EGTA-4 (DMNPE-4) in (*C*) and (*D*)] and was monitored by amperometric detection of quantal monoamine secretion (upper traces). Photolysis was induced within 3 min after the onset of whole-cell perfusion. Traces were obtained from four different cells that were perfused with a pipette solution containing no ATP (*A* and *C*) or 3 mmol/l ATP (Mg^{2+} salt) (*B* and *D*). Mouse pancreatic β-cells and bovine adrenal chromaffin cells were prepared as described (8,9). The times of ultraviolet (UV) radiation–induced photolysis are indicated by arrows. I_{amp}, amperometric current. *A* and *B* are adapted from Takahashi et al. (38) with permission.

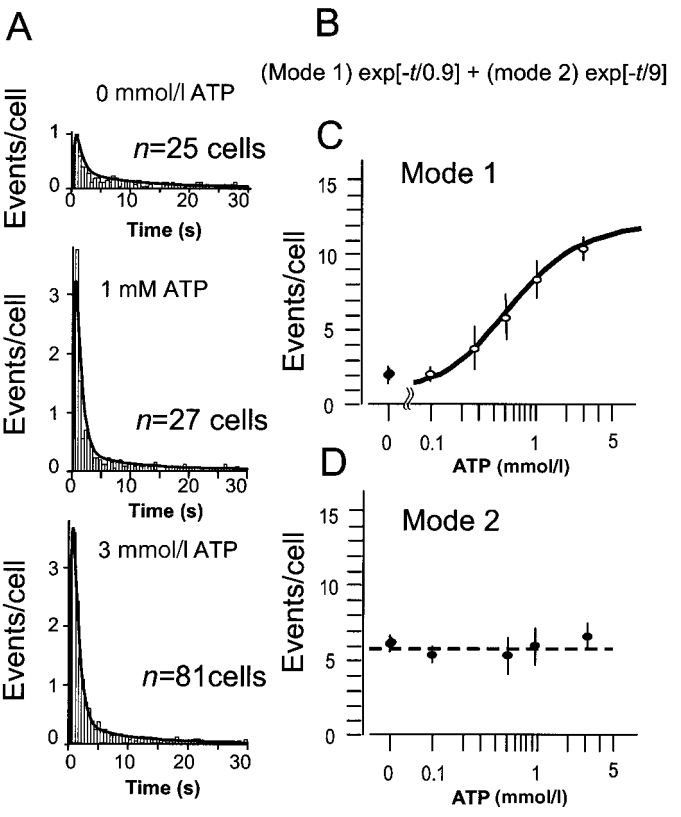

FIG. 2. Dependence of exocytosis in pancreatic β-cells on cytosolic ATP concentration. *A*: Amperometric latency histograms derived from cells that were subjected to whole-cell perfusion with ATP at concentrations of 0, 1, or 3 mmol/l. *B*: Formula that describes the two exponential components of exocytosis. *C* and *D*: Cytosolic ATP dependence of the fast (mode 1) and slow (mode 2) components of exocytosis. The curve in *C* is based on equation (3) with K_{ATP1} = 0.6 mmol/l, K_{ATP2} = 0.15 mmol/l, K_{cAMP} = 4 μmol/l, and *A* = 20 μmol/l. Adapted from Takahashi et al. (38) with permission.

The appearance of amperometric events decays according to two exponential functions in pancreatic β-cells (Fig. 2*B*) (8), reflecting the presence of two pools of insulin granules with distinct time constants for exocytosis. The fast component of exocytosis (mode 1) is selectively potentiated by cytosolic ATP in a concentration-dependent manner (Fig. 2*C* and *D*). The action of ATP was not attributable to an effect on monoamine uptake into secretory granules, because the amplitude of amperometric events was not affected and because ATP-γ-S also facilitated exocytosis (Fig. 3*A*).

Indeed, the effect of ATP-γ-S on exocytosis was more marked than that of ATP itself (Fig. 3*A*). In contrast, another ATP analog, 5′-adenylyl-β,γ-imidodiphosphate (AMP-PNP), was not able to substitute for ATP. Although ATP-γ-S serves as a substrate for PKA and adenylate cyclase, AMP-PNP does not (14), suggesting that one or both of these types of enzyme mediate the action of ATP on exocytosis. We showed that the action of ATP was blocked by the adenylate cyclase inhibitor MDL-12,330A, and that this effect was prevented by the inclusion of cAMP in the patch pipette (8). In addition, the effect of ATP on mode-1 exocytosis was eliminated by the cAMP antagonist adenosine 3′,5′-monophosphothioate (Rp-cAMP) (Fig. 3*B*) and was potentiated by forskolin, an activator of adenylate cyclase (Fig. 3*C*). The action of ATP was also blocked by the PKA inhibitor H89 (Fig. 3*D*). These results thus suggest that the action of ATP is mediated consecutively by adenylate cyclase and PKA. The observation that the effect of ATP-γ-S was greater than that of ATP might be attributable to the relative resistance of thiophosphorylated proteins to phosphatases (15). These results do not exclude a role for a cAMP sensor other than PKA (see below).

To further characterize the mechanism of ATP action, we reinvestigated the ATP requirement for exocytosis in the presence of a saturating concentration (200 μmol/l) of cAMP (8). We confirmed that mode-1 exocytosis is dependent on cytosolic ATP, even in the presence of 200 μmol/l cAMP in the patch pipette (Fig. 4), with the half-maximal concentration being ~0.15 mmol/l (Fig. 5*A*). Our data therefore indicate that the ATP dependence of mode-1 exocytosis reflects a requirement for ATP of both adenylate cyclase and PKA (and, possibly, of other cAMP-dependent mediators).

Mode-1 exocytosis: a model. We have now interpreted our data regarding the action of ATP in mode-1 exocytosis in β-cells with a simple mathematical formula. The cytosolic concentration of cAMP, [cAMP], is related to that of ATP, [ATP], by the equation

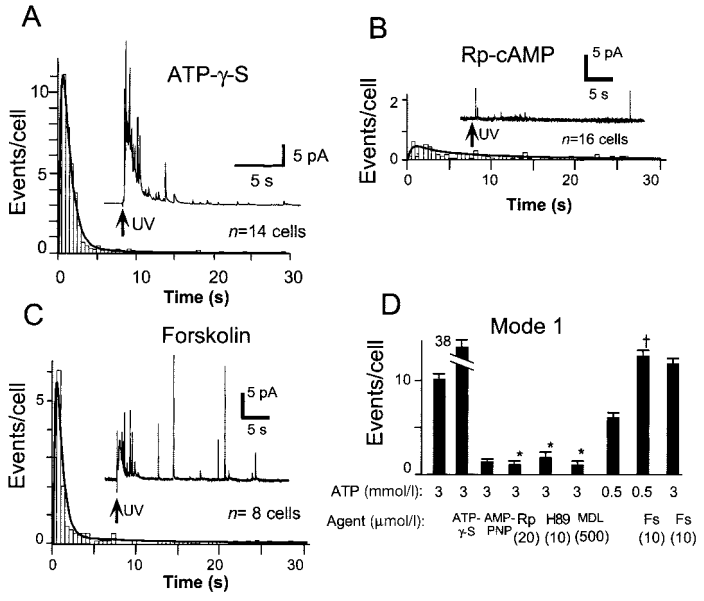

FIG. 3. Role of cAMP in the action of ATP in exocytosis in pancreatic β-cells. *A*: Amperometric latency histogram derived from β-cells that were subjected to whole-cell perfusion with a solution containing 3 mmol/l adenosine-5′-(γ-thio)triphosphate (ATP-γ-S). *B* and *C*: Amperometric latency histograms for β-cells that were subjected to whole-cell perfusion with a solution containing 0.5 mmol/l ATP and treated with either adenosine 3′,5′-monophosphothioate (Rp-cAMP) (20 μmol/l) (*B*) or forskolin (10 μmol/l) (*C*). Insets in *A*, *B*, and *C* are representative amperometric traces for each experimental condition. *D*: Summary of the effects of various agents on mode-1 exocytosis. *P < 0.01 vs. the value for 3 mmol/l ATP; †P < 0.01 vs. the value for 0.5 mmol/l ATP. MDL, MDL-12,330A; Fs, forskolin. Adapted from Takahashi et al. (38) with permission.

$$[\text{cAMP}] = A\frac{\dfrac{[\text{ATP}]}{K_{\text{ATP1}}}}{(1+\dfrac{[\text{ATP}]}{K_{\text{ATP1}}})} \qquad (1)$$

where K_{ATP1} represents the half-maximally effective concentration of ATP (mmol/l) for adenylate cyclase action, and A (μmol/l) represents the rate of cAMP generation (μmol/l per s) divided by the rate of cAMP removal (/s). If cAMP and ATP affect mode-1 exocytosis independently, then the competence of mode-1 exocytosis, E, can be defined by

$$E = \frac{\dfrac{[\text{cAMP}]}{K_{\text{cAMP}}}}{(1+\dfrac{[\text{cAMP}]}{K_{\text{cAMP}}})} \times \frac{\dfrac{[\text{ATP}]}{K_{\text{ATP2}}}}{(1+\dfrac{[\text{ATP}]}{K_{\text{ATP2}}})} \qquad (2)$$

where K_{cAMP} and K_{ATP2} represent the half-maximally effective concentrations of cAMP and ATP for the respective actions of these nucleotides. Combining these two equations gives

$$E = \frac{A\dfrac{[\text{ATP}]}{K_{\text{cAMP}}K_{\text{ATP1}}}}{(1+\dfrac{[\text{ATP}](1+\dfrac{\text{A}}{K_{\text{cAMP}}})}{K_{\text{ATP1}}})} \times \frac{\dfrac{[\text{ATP}]}{K_{\text{ATP2}}}}{(1+\dfrac{[\text{ATP}]}{K_{\text{ATP2}}})} \qquad (3)$$

Our data are well fitted by these equations (Fig. 5*A*, solid lines). The value of K_{ATP2} is estimated as 0.15 mmol/l

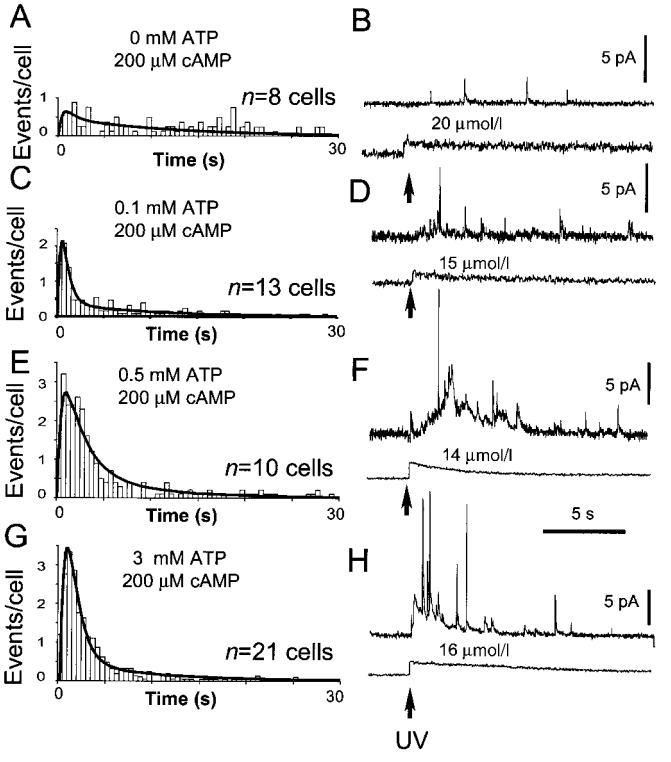

FIG. 4. Effect of ATP on exocytosis in pancreatic β-cells in the presence of exogenous cAMP. *A*, *C*, *E*, and *G*: Amperometric latency histograms derived from β-cells that were subjected to whole-cell perfusion with solutions containing 200 μmol/l cAMP and the indicated concentrations of ATP. Curves are based on the equation shown in Fig. 2*B*. The fast time constant in *E* was adjusted to 2.3 s. *B*, *D*, *F*, and *H*: Representative amperometric (upper) and $[Ca^{2+}]_i$ (lower) traces for the experiments shown in *A*, *C*, *E*, and *G*, respectively. In addition to 200 μmol/l cAMP, patch pipettes contained 10 μmol/l oligomycin in these experiments. UV, ultraviolet. Adapted in part from Takahashi et al. (38) with permission.

based on equation (2) and on the experiments in which a saturating concentration of cAMP (200 μmol/l) was present in the patch pipette (Fig. 5*A*). The value of A is estimated as 20 μmol/l from equation (3) and the data obtained with cells perfused with a solution lacking exogenous cAMP (Fig. 5*A*), assuming K_{cAMP} and K_{ATP1} as 4 μmol/l (15) and 0.6 mmol/l (16), respectively.

This simple theory predicts several key aspects of the regulation of insulin exocytosis observed both in our studies and in those of other laboratories:

Aspect A. Glucose (or cytosolic ATP) supports mode-1 exocytosis even in the absence of hormonal stimulation as a result of the basal activity of adenylate cyclase (Fig. 2*C* and Fig. 5*B*). Equation (3) indicates that the competence of mode-1 exocytosis depends quadratically on [ATP] with two affinity constants,

$$\frac{K_{\text{ATP1}}}{\left(1+\dfrac{A}{K_{\text{cAMP}}}\right)}$$

and K_{ATP2}. Thus, the competence of mode-1 exocytosis is dynamically regulated by glucose.

Aspect B. As a result of the ATP dependence of cAMP action [equation (2)], exocytosis is facilitated by ATP, even in the absence of an increase in cAMP concentration (Fig. 5*A*,*C*). Likewise, mode-1 exocytosis requires ATP even when adenylate cyclase is maximally stimulated.

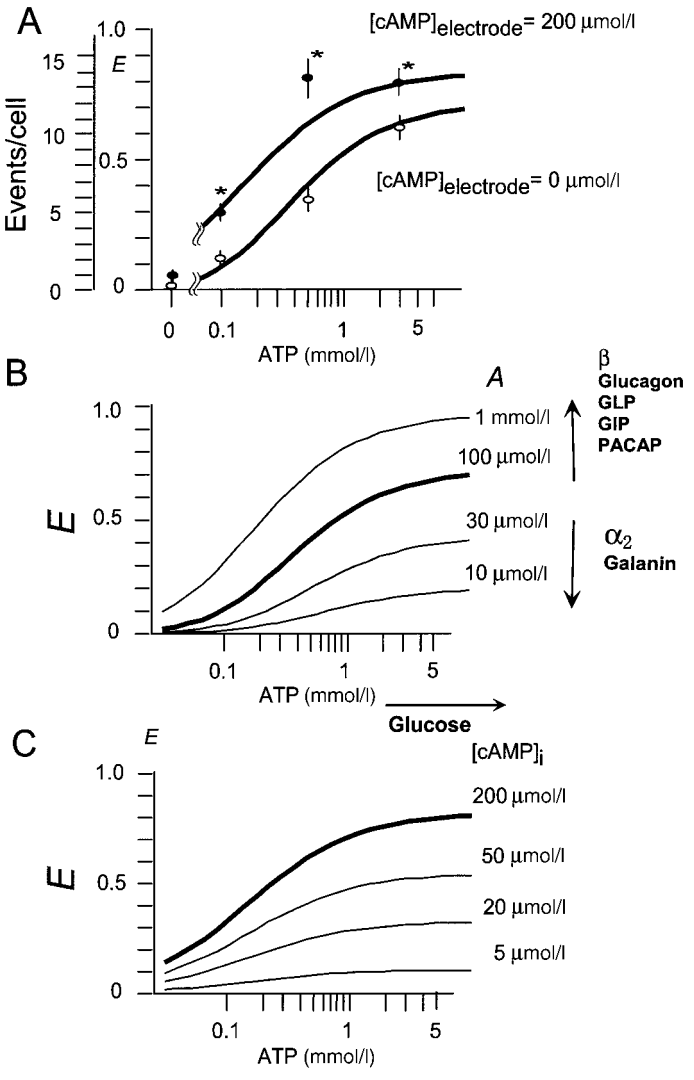

FIG. 5. Dependence of cAMP action in exocytosis on the cytosolic concentration of ATP in β-cells. *A*: Amplitude of mode-1 exocytosis in the absence or presence (200 µmol/l) of cAMP in the patch electrode. The curves are based on equation (2), with [cAMP] = 200 µmol/l, K_{cAMP} = 4 µmol/l, and K_{ATP2} = 0.15 mmol/l, and on equation (3), with A = 20 µmol/l, K_{cAMP} = 4 µmol/l, K_{ATP1} = 0.6 mmol/l, and K_{ATP2} = 0.15 mmol/l. Seventeen exocytotic events per cell are normalized to a value of one for representation of fusion competence, E. The data points for 0 mmol/l ATP were obtained in the presence of 10 µmol/l oligomycin. *B*: Effect of adenylate cyclase activity on mode-1 exocytosis according to equation (3). Ranges of the indicated parameters achieved in the presence of various agents are represented by arrows. GLP, glucagon-like peptide; GIP, glucose-dependent insulinotropic peptide; PACAP, pituitary adenylate cyclase–activating polypeptide; β, β-adrenergic agonists; α_2, α_2-adrenergic agonists. *C*: Effect of ATP on mode-1 exocytosis in the presence of fixed concentrations of cytosolic cAMP according to equation (2).

Aspect C. Hormones that affect the activity of adenylate cyclase effectively regulate mode-1 exocytosis (17) by changing both its sensitivity to ATP,

$$\frac{K_{ATP1}}{\left(1+\frac{A}{K_{cAMP}}\right)}$$

and its amplitude, E [equation (3)].

Aspect D. Mode-1 exocytosis is especially susceptible to hormonal control, because various hormones regulate both cAMP and Ca^{2+} signals (17) and because cAMP and

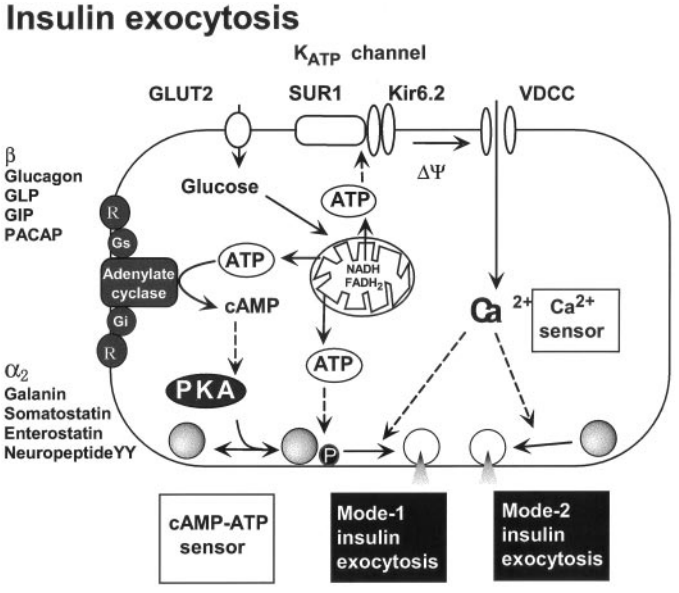

FIG. 6. Schematic representation of glucose signaling in insulin exocytosis. High concentrations of glucose stimulate mitochondrial metabolism and increase the cytosolic concentration of ATP, which is sensed by three distinct components of β-cells: *1*) K_{ATP} channels, which, through their effect on the membrane potential, control voltage-dependent Ca^{2+} channels (VDCCs); *2*) adenylate cyclase, which generates cAMP; and *3*) protein kinase (PKA) and, possibly, other cAMP-dependent proteins. The fast component (mode 1) of exocytosis is regulated in a manner dependent on Ca^{2+} as well as on cAMP and ATP, whereas the slow component (mode 2) depends only on Ca^{2+}. R, receptors coupled to heterotrimeric G proteins (G_s or G_i); ΔΨ, membrane depolarization; $FADH_2$, reduced flavin adenine dinucleotide; GLUT2, glucose transporter 2; SUR1, sulfonylurea receptor 1; Kir, inward rectifier K^+ channel. GLP, glucagon-like peptide; GIP, glucose-dependent insulinotropic peptide; PACAP, pituitary adenylate cyclase–activating polypeptide; β, β-adrenergic agonists; α_2, α_2-adrenergic agonists.

Ca^{2+} affect mode-1 exocytosis independently in a synergic manner (Fig. 6).

Implications of the model of mode-1 exocytosis

Adenylate cyclase. Cytosolic ATP plays an important role in glucose signaling in pancreatic β-cells. Adenylate cyclase thus contributes to glucose-sensing mechanisms (aspect A) in the regulation of insulin exocytosis by cAMP. Indeed, islets of the spiny mouse, which do not exhibit the first phase of glucose-induced insulin secretion, contain an adenylate cyclase that is insensitive to glucose concentration (18). Furthermore, certain diabetic animals have been shown to exhibit reduced cAMP responses to glucose (19,20). Although glucose stimulation does not consistently affect the cytosolic concentration of cAMP (20–24), the production of cAMP by adenylate cyclase may still play a role in preventing a decrease in the concentration of this nucleotide (which might occur as a result of activation of phosphodiesterase, for example) during glucose stimulation. Islets appear to express adenylate cyclase types V and VI (25), whose activity does not depend on an increase in $[Ca^{2+}]_i$. The effect of the inhibitory G protein G_i on adenylate cyclase activity in β-cells is likely important in vivo, where the enzyme is tonically activated by various hormones (26) (Fig. 6). In this regard, it is noteworthy that the inhibitor of the α subunit of G_i was discovered as an islet-activating protein (27).

cAMP sensors. We have previously shown that the effect of cAMP on exocytosis was blocked by the protein kinase

inhibitors (PKI) H89 and H7 (8). Such experiments, however, cannot rule out a role for cAMP other than PKA activation. cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) have recently been implicated in the control of insulin secretion (28,29). Such factors might interact with the sulfonylurea receptor (SUR) in secretory granules (30), which may render GEFs sensitive to ATP, as is the case with K_{ATP} channels.

The requirement of relatively large concentration of ATP in cAMP action is not without precedent. Phosphorylation of the second target site of glycogen synthase by PKA requires a larger concentration of ATP (0.04 to 0.1 mmol/l) than does that of the first site (30,31). Similar large ATP concentrations have also been shown to be required by other kinases (32,33) and adenylate cyclase (31), and may reflect the interaction of ATP with an allosteric binding site (31).

K_{ATP} channel–independent action of glucose on insulin exocytosis. Glucose exerts a stimulatory effect on insulin exocytosis, in addition to its classic action on K_{ATP} channels, which results in an increase in $[Ca^{2+}]_i$ (2,34,35). The effect of ATP in mode-1 exocytosis (aspect A) may contribute to the K_{ATP} channel–independent action of glucose, given that glucose always induces an increase in the cytosolic concentration of ATP. Even though glucose stimulation does not normally result in an increase in the cytosolic concentration of cAMP (24), an increase in cytosolic ATP concentration is suffucient to account for the potentiation of insulin exocytosis by glucose (aspect B). A role for ATP in the K_{ATP} channel–independent action of glucose has previously been suggested by the observation that sodium azide, which reduces the cytosolic ATP concentration, is a potent inhibitor of insulin exocytosis (36,37).

According to this scenario, the K_{ATP} channel–independent effect of glucose on insulin secretion should be dependent on cAMP. Indeed, insulin exocytosis is highly sensitive to cytosolic cAMP, at least in dissociated cells (8,38,39). In whole-islet preparations, however, membrane-permeable antagonists of cAMP (Rp-cAMP) (35,40,41) or of PKA (PKI) (42,43) were shown to have only small effects on glucose-induced insulin secretion. The fact that cumulative insulin secretion was measured after 1 h in these whole-islet studies may have resulted in masking of the most cAMP-sensitive phase of insulin exocytosis. Furthermore, with the use of two-photon excitation imaging, we have shown that membrane-permeable substances (43) may become trapped in the superficial cell layers of pancreatic islets (44) and therefore may give rise to misleadingly small effects. We have also shown with this imaging technique that Rp-cAMP markedly inhibits the first phase of glucose-induced insulin exocytosis in whole-islet preparations (N.T., T. Nemoto, and H.K., unpublished observations).

The observation that cAMP enhances insulin secretion through an action on the K_{ATP} channel–independent pathway of glucose signaling in pancreatic islets (45) is readily explained by aspect C. This previous study also showed that forskolin did not increase insulin exocytosis from islets in the absence of external glucose (45). This lack of an effect of forskolin may be attributable to masking of the effect of cAMP by that of a decrease in ATP concentration (aspect B). The remaining exocytosis apparent under these conditions can also be explained by mode-2 exocytosis, which does not acutely depend on ATP or cAMP.

Mode-1 vs. mode-2 exocytosis. We have investigated Ca^{2+}-dependent exocytosis from β-cells with the use of caged-Ca^{2+} compounds and measured exocytosis within the first 30 s after the photolysis-induced rapid increase in $[Ca^{2+}]_i$. Although our data suggest that mode-2 exocytosis occurs slowly under physiological conditions, they cannot reveal regulatory mechanisms that operate with inherently slower time courses of >30 s. It is likely that mode-2 exocytosis plays a greater role during longer periods of stimulation. The K_{ATP} channel–independent action of glucose also may affect mode-2 exocytosis. Indeed, certain factors are thought to affect predominantly the second phase of glucose-induced insulin secretion (46,47).

It remains unclear how the two modes of insulin exocytosis are assigned to individual insulin granules. It is possible that mode-1 granules are produced as a result of the incorporation of a regulatory component into mode-2 granules. The classical two-compartment model of insulin secretion (48) may also correspond to the two modes of insulin exocytosis. The precise contributions of the two modes of insulin exocytosis to the two phases of insulin secretion await determination by direct examination of this process in intact islets (44) with imaging techniques such as two-photon excitation imaging (49).

ACKNOWLEDGMENTS

We thank Saki Ogawa, Ryosuke Ijwin, and Miho Matsuura for technical assistance. This work was supported by grants to H.K. from the Ministry of Education, Science, Sports, and Culture of Japan, from the Takeda Foundation, from the Human Frontier Science Program, from the Research for the Future Program of the Japan Society for the Promotion of Science, and from CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST), as well as by a grant to N.T. from the Tanabe Medical Frontier Conference. T.S. is an undergraduate student of Tohoku University School of Medicine.

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